Constitutive Activation of Angiotensin II Type-1 Receptor Alters the Orientation of Transmembrane Helix-2

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SUMMARY

A key step in transmembrane (TM) signal transduction by G-protein-coupled receptors (GPCRs) is the ligand-induced conformational change of the receptor, which triggers the activation of a guanine nucleotide-binding protein. GPCRs contain a seven-TM helical structure essential for signal transduction in response to a large variety of sensory and hormonal signals. Primary structure comparison of GPCRs has shown that the second TM helix contains a highly conserved Asp residue which is critical for agonist-activation in these receptors. How conformational changes in TM2 relate to signal transduction by a GPCR is not known because activation-induced conformational changes in TM2 helix have not been measured. Here we use modification of reporter cysteines to measure water accessibility at specific residues in TM2 of the type 1 receptor for the octapeptide hormone angiotensin II. Activation-dependent changes in the accessibility of Cys$^{76}$ on TM2 were measured in constitutively activated mutants. These changes were directly correlated with measurement of function, establishing the link between physical changes in TM2 and function. Accessibility changes were measured at several consecutive residues on TM2 which suggest that TM2 undergoes a transmembrane movement in response to activation. This is the first report of in situ measurement of TM2 movement in a GPCR.
G-protein-coupled receptors (GPCRs)\(^1\) comprise a large family of cell surface receptors that mediate diverse response to a large variety of sensory and hormonal signals (1). They contain a common structural feature characterized by seven transmembrane (TM) helices essential for signal transduction. Although marked variation in the structure of GPCRs is noted, ligand-induced activation of function is thought to involve a common molecular mechanism—rigid body movement of TM helices—which leads to the activation of a specific guanine nucleotide-binding protein (G-protein). Previous studies of rhodopsin and other GPCRs demonstrated that activation induces relative movement of TM3, TM6 and TM7 (2–9).

The type 1 (AT\(_1\)) receptor for the octapeptide hormone angiotensin II (Ang II) is a member of the GPCR family (Fig. 1). It is an important target for drug development, since abnormalities in its function are linked to hypertension, water-electrolyte imbalance, hyperaldosteronism, cardiac hypertrophy and heart failure (10). The AT\(_1\) receptor is coupled to G-protein, G\(_{q/11}\), or in some cases, G\(_i\), G\(_s\) or G\(_{a-12}\). Activation by Ang II results in phospholipase C-activation and inositol phosphate (IP) production, leading to Ang II-specific physiological responses (11, 12). The molecular mechanism of activation of AT\(_1\) receptor has been proposed to involve conformational changes in the TM-domain similar to those in other GPCRs (13). This was based on the finding that Ang II-binding involves interactions with TM3, TM5, TM6 and TM7 (Fig. 1). Interaction of agonist switch residues, Ang II–Tyr\(^4\) with Asn\(^{111}\) and Ang II–Phe\(^8\) with His\(^{256}\) of the AT\(_1\) receptor release the receptor from an inactive state (R), leading to activated state (R\(^*\)) (13–16). Substitution of Asn\(^{111}\) in the middle of TM3 with Gly, Ala, Ser and Cys result in constitutively-activated receptors (14–17). Molecular dynamic simulation studies have predicted that the R\(^*\) state of the AT\(_1\) receptor may require an intramolecular interaction between TM2 and TM7 (17–19). However, the nature of the conformational changes essential for activation of the AT\(_1\) receptor are not known.

In this study, by adopting reporter cysteine accessibility mapping (RCAM) studies (also called SCAM, substituted cysteine accessibility mapping), we found that constitutive activation of AT\(_1\) receptor induces the movement of the TM2 helix. We observed a decrease in the
accessibility of TM2 to water specifically in the constitutively activated mutants of the AT$_1$ receptor, suggesting that activation of receptor lead to changes in water accessibility of TM2.
MATERIALS AND METHODS

Materials—The highly reactive, sulphydryl-specific alkylating reagents used were CH$_3$SO$_2$-SCH$_2$CH$_2$NH$_3^+$ (methanethiosulfonyl ethyl-ammonium [MTSEA$^+$], adduct size about 4.726 Å), CH$_3$SO$_2$-SCH$_2$CH$_2$NMe$_3^+$ (methanethiosulfonyltrimethylammonium [MTSET$^+$], adduct size about 6.058 Å), CH$_3$SO$_2$-SCH$_2$CH$_2$SO$_3^-$ (methanethiosulfonylethyl-sulfonate [MTSES$^-$]) and CH$_3$SO$_2$-SCH (methanethiosulfonyl-methyl), which were purchased from Toronto Research Chemicals, Inc., Ontario, Canada. [Sar$^1$,Ile$^8$]Ang II and [Sar$^1$]Ang II were purchased from Bachem, Torrance, CA. $^{125}$I–[Sar$^1$,Ile$^8$]Ang II, (Sp. Activity 2200 Ci/mmol) was purchased from Dr. Robert Speth, Washington State University, Pulman, WA. Losartan was a gift from DuPont Merck Co., Wilmington, DE.

Mutagenesis and Expression of the AT$_1$ Receptor and Membrane Preparation—The synthetic rat AT$_1$ receptor gene, cloned in the shuttle expression vector pMT-2, was used for expression and mutagenesis, as described in our earlier studies (14–16). To express the AT$_1$ receptor protein, 10 µg of purified plasmid DNA per 10$^7$ cells was used in transfection. COS1 cells (American type culture collection, Rockville, MD), cultured in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), were transfected by the DEAE-dextran method. Transfected cells cultured for 72 h were harvested and cell membranes were prepared by the nitrogen Parr bomb disruption method in the presence of protease inhibitors. The final membrane suspension was at 1 mg/ml protein. The receptor expression was assessed in each case by immunoblot analysis (not shown) and by $^{125}$I–[Sar$^1$,Ile$^8$]Ang II saturation binding analysis.

Radioligand Binding Studies—$^{125}$I–[Sar$^1$,Ile$^8$]Ang II-binding experiments were carried out under equilibrium conditions, as previously described (14–16). Membranes expressing the wild-type (WT) or the mutant receptor were incubated with 300 pM $^{125}$I–[Sar$^1$,Ile$^8$]Ang II for 1 h at 22 °C in a 250 µL volume. Nonspecific binding of the radioligand was measured in the presence of 1 mM $^{127}$I–[Sar$^1$,Ile$^8$]Ang II. The binding experiments were stopped by filtering the binding mixture through Whatman GF/C glass fiber filters, which were extensively washed further with
binding buffer. The bound ligand fraction was determined from the counts per minute (CPM) remaining on the membrane. Binding kinetics were determined using the computer program Ligand®. The $K_d$ and $B_{\text{max}}$ values represent the mean ± S.E.M. of three to five independent determinations.

Reactions with MTS Reagents on Binding—Aliquots of cell membranes (20 µl) were incubated with or without MTS-reagents at the stated concentrations (0.1–12.5 mM) at 22 °C for the indicated time (2–10 min) in 20 mM HEPES buffer (pH 7.4). The reaction mix was then diluted 75-fold with cold buffer to stop the reaction and centrifuged for 10 min at 16,000 $\times$ g at 4 °C, then resuspended in 200 µl. A 150-µl aliquot was used for $^{125}$I-[Sar$^1$,Ile$^8$]Ang II binding analysis. The percent inhibition of $^{125}$I-[Sar$^1$,Ile$^8$]Ang II binding was calculated using the formula $1 - [(\text{specific binding after MTS-reagent}) / (\text{specific binding without reagent})] \times 100\%$. The values represent the mean ± S.E.M. of four to 10 independent determinations.

Inositol Phosphate Formation Studies—Semi-confluent COS1 cells transfected in 60-mm petri dishes were labeled for 24 h with $[^3]$Hmyoinositol (1.5 µCi/ml), specific activity 22 µCi/mol (Amersham), at 37 °C in DMEM containing 10% FBS. The labeled cells were washed three times with Hank’s balanced salt solution (HBSS) and incubated with HBSS containing 10 mM LiCl for 20 min. Agonists were then added and incubation continued for another 45 min at 37 °C. At the end of incubation, the medium was removed, and the total soluble IP was extracted from the cells by the perchloric acid extraction method, as described previously (14–16). The values represent the mean ± S.E.M. of three to four independent determinations.
RESULTS

Experimental Strategy for Mapping Helical Movements—RCAM measures relative changes in water accessibility of site-directed reporter cysteines (Cys) in the inactive and constitutively active states of the AT\textsubscript{1} receptor. Highly reactive, sulphhydryl-specific methanethiosulfonate derivatives (MTS-reagents) were used to modify water-exposed site-directed reporter Cys residues. MTS-reagents are >3000-fold more soluble in aqueous than lipophilic solvents (20). Furthermore, these MTS-reagents react a billion times faster with the thiolate anion species than with the un-ionized thiol (21). Only water accessible thiol groups ionize. Therefore, MTS-reagent’s reaction is specific for water-exposed Cys-sulphhydryl (-SH) groups in the receptor and does not affect buried, lipid-exposed or disulfide-bonded Cys residues. Reaction with MTS-reagents results in the addition of a charged group (either a -sulfonylethylammonium group or a -sulfonylethyl trimethylammonium group) onto the reacted Cys to form a mixed disulfide bond. The modification could block the receptor-ligand interaction either physically (size addition \(\sim\)4Å to 6Å) or via electrostatic repulsion. Because the MTS-reagents are \(\sim\)10 times smaller than Ang II, these reagents are capable of reaching ligand-pocket depths beyond that accessible to Ang II. This mapping method has previously been used in the analysis of several types of integral membrane proteins, ion channels, transporters and other GPCRs (6, 8, 20, 21).

Reaction of the Wild-type AT\textsubscript{1} Receptor with MTS Reagents—Exposure to positively charged MTSEA\textsuperscript{+} and MTSET\textsuperscript{+} reagents for <10 min, abolished specific binding of the antagonist \(^{125}\text{I}\)-[Sar\textsuperscript{1}, Ile\textsuperscript{8}]Ang II to the wild-type AT\textsubscript{1} receptor by nearly 70\% (Fig. 2A). The kinetics of inhibition is dependent on size and concentration of the MTS-reagent and the time of reaction. In contrast, the negatively charged MTSES\textsuperscript{−} and the uncharged methyl-MTS did not inhibit binding. These two reagents reacted poorly, since subsequent reaction with MTSEA\textsuperscript{+} appeared to be normal (data not shown). The inhibition of antagonist binding by MTS-reagents was irreversible and lead to a decrease in the maximal number of binding sites (\(B_{max}\)) without a significant change in binding affinity (Fig. 2B). Dithiothreitol (DTT), commonly used for reversing the MTS-inactivation, could not be used since it inactivates the native AT\textsubscript{1} receptor. In the presence of \(^{125}\text{I}-\)
[Sar\(^1\),Ile\(^8\)]Ang II, the AT\(_1\) receptor was protected against reaction with MTSEA\(^+\) (Fig. 2C) and MTSET\(^+\) (not shown), suggesting that one or more Cys residues in the ligand binding pocket react with MTS-reagents to inhibit antagonist binding. The fast phase of inhibitory reactions with both MTSEA\(^+\) and MTSET\(^+\) are nearly complete in five min (Fig. 2D). The observed B\(_{\text{max}}\) changes appear to be due to the MTS-reaction with Cys residues in the aqueous crevice in the TM domain of the receptor that forms the pocket for Ang II. Similar conclusions have been reached in the study of dopamine and \(\beta_2\)-adrenergic receptors (6).

To identify the MTS-sensitive native Cys residue(s) of the AT\(_1\) receptor, we replaced Cys\(^{76}\) and Cys\(^{289}\) with alanine (Ala). These two residues located in the extracellular half of TM domain are candidates, more likely than the remaining three TM domain Cys residues, for reacting with MTS-reagents (Fig. 1). We also constructed a CYS\(^-\) AT\(_1\) receptor mutant which lacked all non-disulfide bonded Cys residues for comparison. The binding affinity of these three mutants was similar to that of the WT receptor for \(^{125}\)I-[Sar\(^1\),Ile\(^8\)]Ang II (Table I), indicating that Ala substitutions did not affect ligand interaction. In both the WT and C289A mutant receptors, MTSEA\(^+\) inhibited antagonist binding by about 25%. In contrast, binding in the C76A and CYS\(^-\) mutants was resistant to reaction with MTSEA\(^+\) and MTSET\(^+\) (Table I, Fig. 2D). The reaction of WT and these three mutant receptors with MTSET\(^+\) exhibited the same pattern (Fig. 2D). Based on these observations, we conclude that inhibition of [Sar\(^1\), Ile\(^8\)]Ang II binding is due to reaction of MTS-reagents with Cys\(^{76}\) in the TM2 helix and the remaining free Cys residues are not involved.

**Alterations in the Accessibility of Cys\(^{76}\) in Constitutively Activated Mutants**—To map activation-induced conformational changes, we compared the accessibility of the native Cys residues in the WT and gain-of-function mutants. Previous studies demonstrated that the N111S, N111A and N111G mutants of the AT\(_1\) receptor are constitutively activated (14–16). Figure 3 illustrates the finding that the smaller-residue mutations cause a graded elevation of agonist-independent basal intracellular IP accumulation in transfected COS1 cells. In an earlier study we also substituted Asn\(^{111}\) with larger residues (15). For instance, as shown in Fig. 3, Ile\(^{111}\)
substitution suppressed basal activity when compared with the WT. The measured basal activity in the transfected COS1 cells increased linearly with increasing receptor expression. However, there was a progressive increase in basal activity in the mutants in the following rank order from lowest to highest: N111I (slope, 470 ± 40 CPM/ pmol/mg of protein), WT (slope, 850 ± 75 CPM/ pmol/mg of protein), N111A (slope, 1750 ± 280 CPM/ pmol/mg of protein), N111S (slope, 2260 ± 300 CPM/pmol/mg of protein), and N111G (slope, 5870 ± 550 CPM/ pmol/mg of protein). The agonist dependent activity was nearly the same in the WT, N111A, N111S, and N111G mutants (slope, 15550 ± 1500 CPM/ pmol/mg of protein), but was significantly reduced in the N111I mutant (5400 ± 500 CPM/ pmol/mg of protein). The data indicated a progressively increased constitutive activation as the residue size at position 111 of the AT1 receptor decreased. This finding is consistent with previous studies from our laboratory (14–16). The WT and N111I mutant receptors served as controls for the inactive state.

The treatment of the most activated mutant, N111G, with 2.5 mM MTSEA for 2 min did not affect the binding of the radioligand \textsuperscript{125}I-[Sar	extsuperscript{1}, Ile	extsuperscript{8}]Ang II (Fig. 3). Higher concentrations of MTSEA, however, inhibited the binding of \textsuperscript{125}I-[Sar	extsuperscript{1}, Ile	extsuperscript{8}]Ang II to this mutant (Fig. 4). To probe for conformational rearrangements in the series of constitutively activated mutants, MTSEA inhibition of the \textsuperscript{125}I-[Sar	extsuperscript{1}, Ile	extsuperscript{8}]Ang II binding was measured. The following rank order of MTSEA inhibition was found, N111G < N111S < N111A < WT < N111I (Fig. 3). The fractional inhibition caused by 2.5 mM MTSEA correlated significantly with basal activity calculated (r = 0.99, p < 0.05). It is possible to assume that the observed changes in accessibility in these constitutively active mutants arise because of combinatorial changes in accessibility of Cys residues. To evaluate this possibility, we tested double mutants, C76A-N111G, C76A-N111A and C76A-N111I under identical conditions. The double mutants were MTSEA insensitive (Fig. 3), implying that Cys76 is necessary and sufficient for conferring MTSEA inhibition in all mutants. We conclude that the accessibility of Cys	extsuperscript{76} is changed in the constitutively activated state, perhaps due to motion of TM2 helix coupled to the activation of the receptor.
Relative Accessibility of Reporter Cysteines in TM2—In the C76A mutant we substituted TM2 residues, from Ala$^{73}$ to Phe$^{77}$ with Cys, one at a time (Table I). A five-residue stretch is sufficient to infer the ligand-exposed phase, assuming that TM2 adopts $\alpha$-helical conformation. These mutations caused a moderate change in binding affinity for [Sar$^1$, Ile$^8$]Ang II. Reduction in the binding affinity of the nonpeptide antagonist losartan was within 10-fold (data not shown). The $B_{\text{max}}$ values for these mutants varied approximately two-fold. These findings are consistent with preservation of the geometry of the ligand binding pocket. The functional data for the activation of the receptor is given in Table II. Substitution of Ala$^{73}$, Leu$^{75}$, Cys$^{76}$, and Phe$^{77}$ did not significantly alter agonist activation. Substitution of Asp$^{74}$ with Cys hampered agonist activation, although the affinity for the agonist was not significantly altered. The observed defect for the D74C mutation is consistent with the previous reports on the D74A and D74N mutations (19). A similar role in agonist activation is reported in other GPCRs for this highly conserved residue in TM2. When these mutations were combined with the constitutively activated state, the effect on the constitutive activation was consistent with the phenotype displayed by each mutation in the WT receptor (see Table II).

Both MTSEA$^+$ and MTSET$^+$ inhibited the D74C and L75C mutants significantly more than the WT (Cys$^{76}$) (Table I), suggesting that the side chains of the corresponding WT residues are exposed to the ligand pocket. Longer reactions with both MTSEA$^+$ and MTSET$^+$ indicated that D74C is most reactive, followed by L75C and Cys$^{76}$ respectively. The inhibition of D74C is consistent with findings from previous mutagenesis studies that indicate that Asp$^{74}$ faces the ligand pocket in the AT$_1$ receptor (19). In contrast, reaction with MTS reagents did not inhibit antagonist binding to A73C and F77C mutants. Hence, Ala$^{73}$ and Phe$^{77}$ in the WT AT$_1$ receptor very likely face the MTS-inaccessible regions of the AT$_1$ receptor. This finding that the side chains of Ala$^{73}$ ($i$) and Phe$^{77}$ ($i+4$) are MTSEA inaccessible and Asp$^{74}$ ($i+1$) > Leu$^{75}$ ($i+2$) > Cys$^{76}$ ($i+3$) are MTSEA accessible is consistent with $\alpha$-helical conformation for this segment of TM2.
To characterize the predicted TM2 movement in the constitutive activation, the reporter Cys mutants in TM2 were combined with the constitutively activated N111G mutant. The reaction of these mutants with MTSEA\(^+\) and MTSET\(^+\) was compared with the reaction in the WT genetic background (Table I and Fig. 4). The overall accessibility of TM2 residues decrease in the N111G genetic background compared to the WT genetic background. Time-dependent reactions with MTSEA\(^+\) indicated that the accessibility of the reporter Cys\(^{73}\) is increased and that of Cys\(^{76}\) is decreased in the N111G mutant. In the WT genetic background, the Cys\(^{73}\) reporter is inaccessible. The reporters Cys\(^{74}\) and Cys\(^{75}\) are almost equally accessible and Cys\(^{77}\) was inaccessible in both the WT and N111G receptors (Table I and Fig. 4). Short reaction with MTSET\(^+\) did not indicate clear differences, presumably because of larger size of MTSET\(^+\). However, in a 10-min reaction with MTSET\(^+\), reporters Cys\(^{73}\) and Cys\(^{74}\) reacted nearly equally, and Cys\(^{75}\) and Cys\(^{76}\) reacted poorly (Table I, Fig. 4) in the N111G than in the WT genetic background. Thus, reaction with MTSET\(^+\) indicated an additional difference between the WT and constitutively activated states in the orientation of Cys\(^{75}\), which was not clearly indicated in the reaction with MTSEA\(^+\). Therefore, we conclude that the orientation of Ala\(^{73}\), Asp\(^{74}\), Leu\(^{75}\) and Cys\(^{76}\) side chains change in the N111G mutant.
DISCUSSION

The purpose of this study was to obtain insight into the activation mechanism of AT₁ receptor by mapping activation-induced conformational changes using RCAM analysis. Through monitoring the accessibility of native Cys⁷⁶ to conformationally sensitive probes we obtained new evidence that the orientation of TM2 helix changes during constitutive activation of the AT₁ receptor. Reporter Cys residues introduced at certain TM2 positions (74, 75 and 76, but not 73 and 77) reacted with positively charged MTS reagents and inhibited ¹²⁵I-[Sar¹, Ile⁸]Ang II binding to the AT₁ receptor. Comparative analysis of these Cys reporters in the genetic background of WT and gain-of-function mutant, N111G, further established alteration of the TM2 orientation. It is evident from the data obtained that the TM2 region examined here is α-helical in both genetic backgrounds. These findings are consistent with earlier studies, which indicate that RCAM is extremely sensitive to conformational changes and is an effective approach to measuring relative changes in a residue’s position. None of the reporter Cys residues analyzed in this study induced binding defects, showing that elements of secondary and tertiary structure similar to WT are preserved without the formation of non-native intramolecular disulfide bonds. We conclude that the RCAM technique applied to the AT₁ receptor is capable of yielding a realistic map of secondary structure and alterations in receptor conformation.

We speculate that smaller residues substituted for the Asn¹¹¹ induced similar conformational changes that differ only in magnitude. This is based on the observation that different smaller residues substituted for Asn¹¹¹ render the receptor constitutively active, and that the magnitude of constitutive activity correlates with their degree of MTSEA resistance (Fig. 3). Theoretically, however, the observed resistance could be the net result of accessibility changes in multiple native Cys residues. To delineate single versus multiple native cysteine (s) movements, we created the C76A mutant in the Gly¹¹¹ (gain-of-function mutant), Asn¹¹¹(WT) and Ile¹¹¹ (loss-of-function) genetic background and evaluated each double mutant for MTSEA sensitivity. The analysis indicated that C76A mutation rendered AT₁ receptor insensitive to MTSEA in all three functional states (Fig. 3). This suggests that activation-induced changes may not alter the
accessibility of other native cysteines in the AT1 receptor. Thus, the difference in the magnitude of MTSEA sensitivity appears to be merely due to differing degrees of the accessibility of a single native cysteine residue, Cys76. A change in the accessibility of Cys76 reflects movement of the TM2 helix. Using a similar approach, degree of constitutive activity was shown to correlate with degree of MTSEA accessibility of native cysteines in β2-adrenergic receptor (22). The authors suggested that different activating mutations induce a progressive increase in accessibility caused by a progressive conformational change in the helix that carries the reporter Cys residue. Therefore, we conclude that TM2 movement in the AT1 receptor is linked to activation of function.

Activation-induced accessibility of the residues can change not only from transmembrane movement of a residue, but also from rotation of the helix. In general, the magnitude of changes resulting from a helical rotation is small. For example, a decrease in the accessibility of Cys76 combined with an increase in the accessibility of Cys73, Cys74 and Cys75 in the activated state is at least in part consistent with rotational movement of TM2 (Fig. 4). However, our results cannot be attributed solely to the rotation of the helix, because overall reactivity of successive residues Cys74, Cys75 and Cys76 in the activated N111G receptor decreased when compared to the WT receptor. (Fig. 4A). Furthermore, some reporters in the N111G mutant (Cys75 and Cys76) inaccessible to MTSET+ are accessible to the smaller MTSEA+, suggesting that a transmembrane motion of TM2 helix is taking place.

The possibility should also be considered that the observed accessibility changes in TM2 actually reflect a shift in the position of other TM helices that perhaps occurs upon activation of the AT1 receptor (13–16). Although the current study did not directly examine motion of other helices, activation of function may require conformational changes in TM3, TM6 and TM7 helices. TM3 and TM6 helices directly bind agonist switches in Ang II which are critical for activating receptor function (13–16). Motion of TM7 helix may be a participant in activation, since agonist-switch Phe8 of Ang II was shown to photo-crosslink with TM7 (23). However, it is more likely that the motion of TM2 itself is critical during the transition to active-state
conformation and its stabilization because mutation of conserved residues in TM2 cause defects in receptor activation without seriously affecting agonist affinity (18, 19). Such mutations, when combined with constitutively activated mutants, appear to behave independently suggesting lack of interaction in their effects. Thus, AT1 receptor activation perhaps involves motions in TM3, TM6 and TM7 helices which propagate to the TM2 helix and cause accessibility changes.

Based on primary structural homology, five families of GPCRs have been identified and it has been proposed that activation of function involves a common mechanism involving movement of helices (1–8). This was demonstrated by the finding that photoactivation of rhodopsin leads to rigid-body movement of TM3 and TM6 (2, 3). Inhibition of this movement abolished G protein activation. In agreement with these observations, movement of TM3 and TM6 have been observed in β2-adrenergic receptor, α-adrenergic receptor, dopamine receptor and parathyroid hormone receptors (1–8). Similar conformational changes of TM helices are implicated in constitutive activation in several GPCRs. Constitutive activation of GPCRs results from mutation-induced loss of constraints which conform the native receptors to an inactive state in the absence of agonists. Thus, constitutively activating mutations lower thermodynamic barriers for signal activation and apparently also for conformational changes essential for the receptor-activation process. Based on these observations we propose that activating mutations of Asn111 in the AT1 receptor do so by inducing conformational changes similar to those resulting from Ang II-activation.

While movement of TM3, TM6 and TM7 has been implicated in photoactivation of rhodopsin and agonist activation of several other receptors including β2-adrenergic receptor, TM2 movement has thus far not been described for any receptor. It is known that TM2 contains one of the most conserved motifs, NLxADL, which is implicated in agonist-induced signaling in the GPCR family (Fig. 1) (1, 24, 25). An essential role for significant agonist-induced structural changes in TM2 of the AT1 receptor has been proposed. Substitution of the Asp74 residue in the AT1 receptor leads to defective G-protein activation but has no effect on ligand binding (18, 19). It was proposed that TM2 and TM7 helices move closer to each other in the
activated state and establish an interaction between Asp\textsuperscript{74} in TM2 and Tyr\textsuperscript{292} in TM7 (17, 18).

The mutational effects of Asp\textsuperscript{74} and the predicted conformational changes in TM2 are consistent with the well-documented role for this residue in a majority of other GPCRs (24, 25). Thus, an essential conformational change in TM2 is very likely an important part of the coordinated movement of TM helices that constitute the general GPCR activation mechanism. The prime objective of the present study was to detect conformational changes in TM2 residues experimentally using RCAM studies, and to further examine their dynamics and possible involvement in receptor function. Although a detailed structural interpretation cannot be made from this study, it is clear that significant structural changes take place in the TM2 helix upon receptor activation. A prominent signature of the activated receptor conformation is the decreased reactivity of Cys\textsuperscript{76}. Perhaps the absence of structural constraints allows flexibility to this TM2 region in the native receptor state such that Cys\textsuperscript{76} can sample a larger reactive volume. A conformational change, such as the one induced by the N111G mutation (or by interaction of Asn\textsuperscript{111} with Tyr\textsuperscript{4} of Ang II during ligand activation), can then induce motion and a change in the orientation of TM2 that moves Cys\textsuperscript{76} farther from the water-filled ligand pocket. At the same time the motion of residues Ala\textsuperscript{73}, Asp\textsuperscript{74} and Leu\textsuperscript{75} is consistent with a rigid-body motion of TM2. Given the importance of the highly conserved Asp\textsuperscript{74} in the function of several GPCRs (24, 25), the motion of this residue may be relevant to finding a general mechanism for receptor activation. Elucidation of the AT\textsubscript{1} receptor activation mechanism and its functional importance is the goal of our future work.
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REFERENCES


FOOTNOTES

1 Abbreviations: Ang II, angiotensin II [Asp\textsuperscript{1}-Arg\textsuperscript{2}-Val\textsuperscript{3}-Tyr\textsuperscript{4}-Ile\textsuperscript{5}-His\textsuperscript{6}-Pro\textsuperscript{7}-Phe\textsuperscript{8}-COO\textsuperscript{-}]; AT\textsubscript{1}, angiotensin II type-1; RCAM, reporter cysteine accessibility mapping; MTSEA, methanethiosulfonyl ethyl-ammonium; MTSET, methanethiosulfonyl trimethyl-ammonium; TM, transmembrane; IP, inositol phosphate; SH, sulfhydryl group; R, the basal inactive state; R', intermediate activated state; R*, fully activated state; WT, wild-type
FIGURE LEGENDS

Fig. 1. A secondary structure model of rat AT1 receptor revised based on the structure of bovine rhodopsin (27). The Cys residues in the native receptor are highlighted. Residues that have been shown to interact with Ang II are indicated by ♦. The sites on TM2 examined in this study are shown within a box. The residues 69–75 (–NLALADL–) comprise one of the most conserved motifs (NLAXxADL) in the GPCR family (24). Extracellular Cys\textsuperscript{18}–Cys\textsuperscript{274} and Cys\textsuperscript{101}–Cys\textsuperscript{180} form disulfide bonds (26), the cytoplasmic Cys\textsuperscript{355} may be palmitoylated, and five Cys residues in the TM domain are thought to be free thiols.

Fig. 2. (A) Inhibition of specific binding of \textsuperscript{125}I-[Sar\textsuperscript{1},Ile\textsuperscript{8}]Ang II to the WT AT\textsubscript{1} receptor after treatment with MTS reagents (0.1–12.5 mM) for 2–10 min. (B) Scatchard plot analysis of binding with or without MTSEA\textsuperscript{+} reaction. (C) Ligand protection of AT\textsubscript{1} receptors against MTSEA\textsuperscript{+} inactivation. Specific binding remaining after the \textsuperscript{125}I-[Sar\textsuperscript{1},Ile\textsuperscript{8}]Ang II-bound receptor (100%, ~28,000 CPM) was exposed to 2.5 mM MTSEA\textsuperscript{+} for 15 min at 37 °C. (D) Inhibition of specific binding to the WT receptor, and mutants after 2-min reactions with MTSEA\textsuperscript{+} or MTSET\textsuperscript{+}. The intrinsic rate of MTSET\textsuperscript{+} reaction with free thiols in solution was 2.5-fold higher than that with MTSEA\textsuperscript{+} (16). However, MTSEA\textsuperscript{+} inhibition greater than MTSET\textsuperscript{+} in a protein is due to the difference in the size of the head groups of the two reagents, which imposes steric restriction. The size of the –N(CH\textsubscript{3})\textsubscript{3}\textsuperscript{+} group is about 6.058Å in MTSET\textsuperscript{+} compared with the size of –NH\textsubscript{3}\textsuperscript{+} (about 4.726Å) in MTSEA\textsuperscript{+}. Values of two to five independent experiments (mean ± S.E.M.) are shown.

Fig. 3. Correlation between accessibility of Cys\textsuperscript{76} (●) and the constitutive activity in the N111G, N111S, N111A, WT and N111I AT\textsubscript{1} receptors (○). IP production without agonist activation is measured as the percentage of IP produced by the same receptor when stimulated with 1 μM [Sar\textsuperscript{1}]Ang II (100%) in parallel. The values were calculated from slope of basal IP as a function of receptor density obtained from 4 individual transfections. The slopes of the lines obtained for
each mutant are given in the text. The observed relationship between basal activity and surface area of the side chain present at position 111 has previously been described in detail earlier (15). MTSEA\(^+\)-inhibition of specific binding of \(^{125}\)I-[Sar\(^1\), Ile\(^8\)]Ang II was measured as described under Materials and Methods. It represents the accessibility of Cys\(^{76}\) (\(r = 0.99, n = 5, y = -31.4 + 0.6\ X + 0.016\ X^2\)). The data are given as fractional inhibition compared with control binding activity without MTSEA treatment (means ± S.E., \(n = 5\)). The correlation of fractional inhibition of specific \(^{125}\)I-[Sar\(^1\), Ile\(^8\)]Ang II binding produced by MTSEA with the % of the constitutively activated IP formation in the mutants was plotted. Both parameters best fit polynomial function using the least-squares error method (\(r = 0.99, n = 5, y = 240 - 4.4\ X + 0.028\ X^2 - 6.16\times 10^{-5}\ X^3\)).

The MTSEA reaction with C76A-N111G, C76A-WT and C76A-N111I receptors is shown as ■.

Fig. 4. (A) Relative accessibility to MTSEA\(^+\) and MTSET\(^+\) of reporter Cys in the WT and N111G genetic background (means ± S.E., \(n = 5\)). The inhibition in the C76A mutant in each genetic background is used as zero inhibition to calculate relative inhibition caused by the reaction of each reporter Cys. The symbols *, indicate significant increase (\(P < 0.05\)) and † indicates significant decrease (\(P < 0.05\)) compared to WT genetic background.
Table I

Ligand-affinity, $B_{\text{max}}$ and inhibition by MTS reagents of wild-type and reporter Cys mutant AT$_1$ receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (pmol/mg)</th>
<th>% Inhibition of Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MTSEA$^+$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 min</td>
</tr>
<tr>
<td>(Cys$^{76}$/WT</td>
<td>0.37 ± 0.07</td>
<td>4.5 ± 0.3</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>A73C, C76A</td>
<td>4.20 ± 1.30</td>
<td>5.1 ± 0.2</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>D74C, C76A</td>
<td>3.47 ± 0.14</td>
<td>3.9 ± 0.5</td>
<td>43 ± 5*</td>
</tr>
<tr>
<td>L75C, C76A</td>
<td>1.17 ± 0.24</td>
<td>4.5 ± 0.6</td>
<td>36 ± 3*</td>
</tr>
<tr>
<td>C76A</td>
<td>0.87 ± 0.11</td>
<td>3.9 ± 0.5</td>
<td>8 ± 4†</td>
</tr>
<tr>
<td>C76A, F77C</td>
<td>0.64 ± 0.25</td>
<td>4.5 ± 0.3</td>
<td>4 ± 1†</td>
</tr>
<tr>
<td>C289A</td>
<td>0.39 ± 0.02</td>
<td>5.6 ± 0.4</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>CYS$^*$</td>
<td>1.07 ± 0.08</td>
<td>5.7 ± 0.2</td>
<td>6 ± 3†</td>
</tr>
<tr>
<td>(Cys$^{76}$/N111G</td>
<td>0.36 ± 0.02</td>
<td>3.6 ± 0.1</td>
<td>4 ± 4†</td>
</tr>
<tr>
<td>(Cys$^{76}$/N111S</td>
<td>0.20 ± 0.01</td>
<td>4.9 ± 0.1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>(Cys$^{76}$/N111A</td>
<td>0.51 ± 0.03</td>
<td>6.0 ± 0.2</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>(Cys$^{76}$/N111I</td>
<td>0.44 ± 0.13</td>
<td>3.1 ± 0.1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>C76A/N111G</td>
<td>0.28 ± 0.04</td>
<td>4.6 ± 0.4</td>
<td>11 ± 3†</td>
</tr>
<tr>
<td>C76A/N111I</td>
<td>0.38 ± 0.04</td>
<td>3.9 ± 0.4</td>
<td>6 ± 3†</td>
</tr>
<tr>
<td>A73C, C76A/N111G</td>
<td>0.31 ± 0.02</td>
<td>5.2 ± 0.4</td>
<td>24 ± 4†</td>
</tr>
<tr>
<td>D74C, C76A/N111G</td>
<td>0.77 ± 0.06</td>
<td>3.0 ± 0.3</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>L75C, C76A/N111G</td>
<td>0.42 ± 0.03</td>
<td>3.9 ± 0.1</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>C76A, F77C/N111G</td>
<td>0.23 ± 0.01</td>
<td>5.6 ± 0.4</td>
<td>5 ± 2†</td>
</tr>
</tbody>
</table>

N.D.: Not Determined at this time.

The irreversible inhibition of the $^{125}$I-(Sar$^1$,Ile$^8$)Ang II-binding of WT and mutant receptors resulting from reaction with 2.5 mM MTSEA$^+$ and 2.5 mM MTSET$^+$ for indicated time. The inhibition is calculated as described in the Methods section. The values shown are means and standard errors for 4–10 independent determinations for each mutant. Analysis of variance showed * significant increase (p < 0.05), and † significant decrease (p < 0.05), in the reaction with MTSEA$^+$ and MTSET$^+$ compared to WT. The differences between the means were evaluated by the most significant difference procedure. In all experiments an excess of $^{125}$I(Sar$^1$,Ile$^8$)Ang II was used to obtain > 99% receptor in the ligand-bound form. The total specific binding in each reaction was within 10% of total CPM added, and the nonspecific binding was < 5% of specific binding.
### Table II

*Effect of Cys substitution of TM2 residues on function*

<table>
<thead>
<tr>
<th>TM2-mutation</th>
<th>TM3-mutation</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None(Asn(^{111}))</td>
<td>Basal</td>
<td>Agonist</td>
<td>Basal</td>
<td>Agonist</td>
</tr>
<tr>
<td>None</td>
<td>6 ± 2</td>
<td>100 ± 5</td>
<td>42 ± 2</td>
<td>104 ± 7</td>
<td></td>
</tr>
<tr>
<td>A73C, C76A</td>
<td>6 ± 3</td>
<td>70 ± 6</td>
<td>26 ± 2</td>
<td>88 ± 4</td>
<td></td>
</tr>
<tr>
<td>D74C, C76A</td>
<td>5 ± 4</td>
<td>7 ± 3</td>
<td>19 ± 3</td>
<td>18 ± 3</td>
<td></td>
</tr>
<tr>
<td>L75C, C76A</td>
<td>5 ± 2</td>
<td>93 ± 3</td>
<td>42 ± 3</td>
<td>97 ± 6</td>
<td></td>
</tr>
<tr>
<td>C76A</td>
<td>3 ± 3</td>
<td>95 ± 8</td>
<td>39 ± 3</td>
<td>99 ± 5</td>
<td></td>
</tr>
<tr>
<td>C76A, F77C</td>
<td>5 ± 1</td>
<td>98 ± 1</td>
<td>58 ± 4</td>
<td>135 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

*The values represent total inositol phosphates measured in COS1 cells transfected with WT and mutant AT\(_1\) receptor expression plasmids (10 µgDNA/10\(^7\) cells) in three independent experiments. IP value measured in COS1 cells transfected with expression plasmid without the AT\(_1\) receptor gene (< 0.2% compared to activated WT) was subtracted from all values. The average of agonist-stimulated (by 10\(^{-6}\)M [Sar\(^1\)]Ang II) IP values for the WT AT\(_1\) receptor was 10,000 ± 400 CPM/p.mol receptor. This value was taken as 100% to represent the effect of individual Cys substitution on the function of WT and the N111G mutant AT\(_1\) receptor. The expression level of receptor estimated for different mutants varied within 2-fold.*
Figure 2

A.

B.

C.

D.
Figure 3

![Graph showing % Basal IP Production and % Inhibition of 125I-[Sar1,Ile8] Ang II Binding with different residues at position 111.](image-url)
Figure 4

![Graph showing activation-induced conformation changes of AT1 receptor](image-url)
Constitutive activation of angiotensin II type-1 receptor alters the orientation of transmembrane helix-2
Shin-ichiro Miura and Sadashiva S. Karnik

J. Biol. Chem. published online April 30, 2002

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